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Description

Field of the invention

This invention relates to the production, via recombinant DNA technology, of useful polypeptides in *Saccharomyces cerevisiae* (yeast), and to the means and methods of such production.

Background of the invention

The publications and other materials referred to herein to illuminate the background of the invention and, in particular cases, to provide additional detail respecting its practice are incorporated herein by reference, and, for convenience, are numerically referenced and grouped in the appended bibliography.

Recombiant DNA technology

With the advent of recombinant DNA technology, the controlled microbial production of an enormous variety of useful polypeptides has become possible. Already in hand are bacteria modified by this technology to permit the production of such polypeptide products as somatostatin (1), the component A and B chains of human insulin (1), human proinsulin (2), thymosin alpha 1 (3), human growth hormone (4), human (5) and hybrid (6) leukocyte and fibroblast (7) interferons, as well as a number of other products. The continued application of techniques already in hand is expected in the future to permit bacterial production of a host of other useful polypeptide products, including other hormones, enzymes, immunogens useful in the preparation of vaccines, immune modulators and antibodies for diagnostic and drug-targeting applications.

The workhorse of recombinant DNA technology is the plasmid, a non-chromosomal loop of double-stranded DNA found in bacteria and other microbes, oftentimes in multiple copies per cell. Included in the information encoded in the plasmid DNA is that required to reproduce the plasmid in daughter cells (i.e., an "origin of replication") and, ordinarily one or more selection characteristics such as, in the case of bacteria, resistance to antibiotics, which permit clones of the host cell containing the plasmid of interest to be recognized and preferentially grown under selective conditions. The utility of plasmids lies in the fact that they can be specifically cleaved by one or another restriction endonuclease or "restriction enzyme", each of which recognizes a different site on the plasmid DNA. Thereafter heterologous genes or gene fragments may be inserted into the plasmid by endwise joining at the cleavage site or at reconstructed ends adjacent to the cleavage site. DNA recombination is performed outside the cell, but the resulting "recombinant" plasmid can be introduced into it by a process known as transformation and large quantities of the heterologous gene-containing recombinant plasmid are then obtained by growing the transformant. Moreover, where the gene is properly inserted with reference to portions of the plasmid which govern the transcription and translation of the encoded DNA information, the resulting expression vehicle can be used to actually produce the polypeptide sequence for which the inserted gene codes, a process referred to as expression.

Expression is initiated in a region known as the promoter which is recognized by and bound by RNA polymerase. The polymerase travels along the DNA, transcribing the information contained in the coding strand from its 5' to 3' end into messenger RNA which is in turn translated into a polypeptide having the amino acid sequence for which the DNA codes. Each amino acid is encoded by a nucleotide triplet or "codon" within what may for present purposes be referred to as the "structural gene", i.e., that part which encodes the amino acid sequence of the expressed product. After binding to the promoter, the RNA polymerase, transcibes a 5' leader region of messenger RNA, then a translation initiation or "start signal" (ordinarily ATG, which in the resulting messenger RNA becomes AUG), then the nucleotide codons within the structural gene itself. So-called stop codons are transcribed at the end of the structural gene whereafter the polymerase may form an additional sequence of messenger RNA which, because of the presence of the stop signal, will remain untranslated by the ribosomes. Ribosomes bind to the binding site provided on the messenger RNA, and themselves produce the encoded polypeptide, beginning at the translation start signal and ending at the previously mentioned stop signal. The resulting product may be obtained by lysing the host cell and recovering the product by appropriate purification from other microbial protein or, in particular instances, possibly by purification from the fermentation medium into which the product has been secreted.

Plasmids employed in genetic manipulations involved in the construction of a vehicle suitable for the expression of a useful polypeptide product are referred to as DNA transfer vectors. Thus, employing restriction enzymes and associated technology, gene fragments are ordered within the plasmid in *in vitro* manipulations, then amplified *in vivo* in the transformant microbes into which the resulting, recombinant plasmid has been 'transferred'. A "DNA expression vector" comprises not only a structural gene intended for expression but also a promoter and associated controls for effecting expression from the structural gene. Both transfer and expression vectors include origins of replication. Transfer vectors must and expression vectors may also include one or more genes for phenotypic selection of transformant colonies.

Thus far, the useful products of expression from recombinant genes have fallen into two categories. In the first, a polypeptide having the amino acid sequence of a desired end product is expressed directly, as in the case of human growth hormone and the interferons referred to above. In the second, the product of expression is a fusion protein which includes not only the amino acid sequence of the desired end product

but also one or more additional lengths of superfluous protein so arranged as to permit subsequent and specific cleavage away of the superfluous protein and so as to yield the desired end product. Thus, cyanogen bromide cleavage at methionine residues has yielded somatostatin, thymosin alpha 1 and the component A and B chains of humun insulin from fusion proteins; enzymatic cleavage at defined residues has yielded beta endorphin (8);

A "biocompetent polypeptide", as that term is used herein, refers to a product exhibiting bioactivity akin to that of a polypeptide innately produced within a living organism for a physiological purpose, as well as to intermediates which can be processed into such polypeptides, as by cleavage away of superfluous protein, folding, combination (as in the case of the A and B chains of human insulin), etc.

Saccharomyces cerevisiae

The cells of *Saccharomyces cerevisiae*, or yeast, are, like those of mammalian organisms, eukaryotic in nature as distinguished from the prokaryotic nature of bacteria. With regard to mechanisms for the expression of genetic information, eukaryotes are distinguished from bacteria by:

(1) Chromosomes which are organized in 140 base pair units, each containing two molecules each of histones H2A, H2B, H3, and H4.

(2) Transcription of the protein-encoding gene by the alpha-amanitin sensitive RNA polymerase II.

(3) Post transcriptional addition of Gppp and polyadenylic acid to the 5' and 3' termini of mRNA molecules.

(4) Transport of newly completed mRNA from the nuclei where they are transcribed to the cytoplasm where they are translated.

(5) Some but not all eukaryotic genes contain intervening sequences (introns) which make them non-colinear with the corresponding mature mRNA molecule. The initial transcription products of these genes contain the intron sequence which is spliced out subsequently in the formation of a finished mRNA molecule.

The nucleotide sequences of all eukaryotic cells are transcribed, processed, and then translated in the context described above. There are reasons to believe that expression of eukaryotic genes may proceed with greater efficiency in yeast than in *E. coli* because yeast is a eukaryote cell.

A number of workers have previously expressed, or attempted to express, foreign genes in yeast transformants. Thus, attempted expression from a fragment comprising both a promoter and structural gene for rabbit globin is reported (9) to have yielded partial mRNA transcripts, seemingly unaccompanied either by translation into protein or maturation (intron elimination) of the message. A gene coding for Drosophila GAR tranformylas (yeast *ADE8*), an enzyme in the adenine synthesis pathway, is reported to have been expressed under the control of its own promoter (10). A number of yeast proteins have hitherto been expressed in yeast via recombinant plasmids (see, e.g., 12). In the experiments, as in the Ade-8 case earlier discussed, expression occurred under the selective pressure of genetic complementation. Thus, each expression product was required for growth of the host strains employed, mutants whose chromosomal DNA was defective in the structural gene(s) from which expression occurred.

The availability of means for the production in yeast of proteins of choice could provide significant advantages relative to the use of bacteria for the production of polypeptides encoded by recombinant DNA. Yeast has been employed in large scale fermentations for centuries, as compared to the relatively recent advent of large scale *E. coli* fermentation. Presently, yeast can be grown to higher densities than bacteria, and is readily adaptable to continuous fermentation processing. Many critical functions of the organism, e.g., oxidative phosphorylation, are located within organelles, and hence not exposed to the possible deleterious effects of the organism's overproduction of foreign proteins. As a eukaryotic organism, yeast may prove capable of glycosylating expression products where important to enhanced bioactivity. Again, it is possible that as eukaryotic organisms, yeast cells will exhibit the same codon preferences as higher organisms, tending toward more efficient production of expression products from mammalian genes or from complementary DNA (cDNA) obtained by reverse transcription from, e,g., mammalian messenger RNA. Until the present invention, however, attempts to produce biocompetent expression products other than those required for cellular growth have proven largely unsuccessful.

Brief summary of the invention

The present invention provides DNA expression vectors capable, in transformant strains of yeast, of expressing biologically competent (preferably pharmacologically active) polypeptides under the control of genetically distinct yeast promoters, the polypeptides being ordinarily exogenous to yeast and other than those required for growth of the transformant. The invention also provides DNA transfer vectors for the transformation of yeast strains with genes encoding biocompetent polypeptides, as well as novel yeast organisms and cultures thereof incorporating such vectors and methods for the formation of the same. The structural genes incorporated in the expression vectors and transformant organisms of the invention are under the control of genetically distinct yeast promoters, i.e. promoters different from those evolutionarily associated with the subject structural genes.

According to one aspect of the present invention there is provided a DNA vector suitable for use in expressing exogenous genes in yeast, comprising a sequence which is replicable in yeast, a 5' flanking sequence of a yeast structural gene including a promoter, a site downstream of said 5' flanking sequence in

the direction of transcription for insertion of a structural gene coding for a polypeptide ordinarily exogenous to yeast so as to be transcribable under the control of said promoter and translatable from a start signal, and a sequence allowing phenotypic selection of yeast transformants.

The invention also provides a recombinant DNA vector for use in expressing an exogenous structural gene in a suitable yeast strain, comprising a DNA vector as described above and a said exogenous gene inserted at said site so as to be transcribable under the control of said promoter and translatable from a start signal.

The invention further includes yeast strains transformed with such a recombinant DNA vector, a method of forming such transformed yeast strains, and a method of producing a biocompetent polypeptide therefrom.

In another aspect, therefore, the present invention provides a method of producing a desired heterologous polypeptide in yeast by culturing a yeast strain transformed with a recombinant DNA expression vector replicable in said yeast strain, characterised in that the vector contains an exogenous DNA sequence coding for the polypeptide transcriptionally downstream of a 5' flanking sequence of a yeast structural gene containing a promoter which is functional in said yeast strain, and a translation initiation signal between said promoter and the exogenous coding sequence, so that the exogenous sequence is transcribed from said promoter and translated from said translation initiation signal.

The manner in which these and other objects and advantages of the invention are obtained will become apparent from the detailed description which follows, and from the accompanying drawings in which:

Figure 1 schematically illustrates the construction of a DNA transfer vector having a single *Eco* RI restriction site for the subsequent insertion of a yeast promoter and comprising both bacterial and yeast origins of replication and selection phenotypes;

Figure 2 schematically illustrates the construction of alcohol dehydrogenase promoter fragments for insertion into the transfer vector of Figure 1;

Figure 3 illustrates the coding strand sequence and end points (904, 906, etc.) of a series of yeast promoter fragments made by digestion with the exonoclease *Bal* 31; and attachment of *Eco*RI molecular recombinational linkers.

Figure 4 schematically illustrates the insertion of yeast promoter fragments into the transfer vector of Figure 1 and subsequent insertion (in two orientations) of a structural gene encoding human leukocyte interferon D.

In the Figures and throughout, the letters A, T, C and G respectively connote the nucleotides containing the bases adenine, thymine, cytosine and guanine. Only the coding strands of plasmids and gene fragments are depicted. Though obviously not to scale, the representations of plasmids depict the relative position of restriction enzyme cleavage sites ("*Eco*RI", "*Hind*III" etc.) and other functions such as tetracycline resistance ("Tc"") and ampicillin resistance ("Ap"").

Description of the preferred embodiments

Preferred embodiments of the invention are obtained by bringing an exogenous gene under the control of a yeast promoter carried by a plasmid suitable for the transformation of yeast. Essentially any yeast strain suited for the selection of transformants may be employed. In order to achieve direct expression of the desired end product or an intermediate therefor, rather than a fusion comprising portions of the yeast protein whose expression is controlled by the promoter in wild-type strains, the parental plasmid is resected toward the promoter in the direction opposite that of transcription, so as to excise the ATG triplet which initiates translation of mRNA encoding the yeast protein referred to.

An ordinarily exogenous gene, with its associated start signal, may then be inserted at the endpoint of the resection, and thus positioned for direct expression under the control of the yeast promoter. This and other aspects of the invention are illustrated in the description of preferred embodiments which follow.

Methods

Materials: All DNA restriction and metabolism enzymes were purchased from New England and Biolabs except for exonuclease *Bal* 31 and bacterial alkaline phosphatase, which were obtained from Bethesda Research Laboratories. DNA restriction enzyme and metabolic enzymes were used in conditions and buffers described by the respective manufacturers. ATP and the deoxynucleoside triphosphates dATP, dGTP, dCTP and dTTP were purchased from PL Biochemicals. *Eco* RI, *Bam* HI, *Hind* III and *Xho* I linkers were obtained from Collaborative Resarch, Inc. [α-^{32}P] was obtained from New England Nuclear Corp.

DNA Preparation and Transformation: Purification of covalently closed circular plasmid DNAs from *E. coli* (13) and yeast (14) plus the transformation of *E. coli* (15) was as previously described. Transformation of yeast was as described by Hsiao and Carbon (16) with the exception that 1.2 M Sorbitol was used instead of 1.0 M Sorbitol. *E. Coli* miniscreens were as described by (17).

Strains and Media: *E. coli* strain JA300 (*thr leu*B6 *thi thy*A *trp*C1117 *hsd*M$^-$ *hsd*R$^-$ str^R) (18) was used to select for plasmids containing functional *trp1* gene. *E. coli* K-12 strain 294 (ATCC No. 31446, deposited 28 Oct. 1978) (19) was used for all other bacterial transformation. Yeast strains RH218 having the genotype (a *trp*l *gal*2 *suc*2 *mal* CUPI) (20) and GM-3C-2 (α, *leu* 2—3, *leu* 2—112, *trp* 1—1, *his* 4—519, *cyc* 1—1, *cyp* 3—1)

(21) were used for yeast transformations. Yeast strain RH 218 was deposited without restriction in the American Type Culture Collection, ATCC No. 44076 on 8 Dec. 1980.

M9 (minimal medium) with 0.25 percent casamino acids (CAA) and LB (rich medium) were as described by Miller (22) with the addition of 20 µg/ml ampicillin (Sigma) after media is autoclaved and cooled. Yeast were grown on the following media: YEPD contained 1 percent yeast extract, 2 percent peptone and 2 percent glucose ±3 percent Difco agar. YNB+CAA contained 6.7 grams of yeast nitrogen base (without amino acids) (YNB) (Difco), 10 mg of adenine, 10 mg of uracil, 5 grams CAA, 20 grams glucose and ±30 grams agar per liter. The selection of ADH promoter active fragments occurred on YEPGE plates containing 3 percent glycerol and 2 percent ethanol substituted for glucose in the YEPD formula. Leucine prototrophy was determined on plates containing 6.7 gms YNB, 20 gms glucose, 50 mgs histidine and 50 mgs trytophan and 30 gms Difco agar per L.

Construction and identification of ADH promoter deletions (Fig. 2): pY9T6 was digested with Sau3A then run on a preparative 1 percent agarose gel. The 1600 bp fragment containing the ADH promoter region was cut from the gel, electroeluted then purified on a diethylamino cellulose (DE52, Whatman) column before ethanol precipitation. Fragment DNA was resuspended in DNA Polymerase I (Klenow fragment) buffer supplemented with the four deoxyribonucleoside triphosphates in a final concentration of 80 µM. Polymerase I was added and the thirty-minute room temperature reaction was terminated by ethanol precipitation of the DNA. An equal molar amount of *Bam*HI and *Hind*III linker was added to the resuspended Sau3A fragment so that each linker was in a 20:1 molar excess to the large DNA fragment. T_4 DNA ligase was added and the 12 hour reaction occurred at 12 degrees centrigrade. After ethanol precipitation and resuspension in the appropriate buffer, the DNA was digested with *Bam*HI, then *Hind*III. The now larger promoter-containing fragment was purified away from the unattached linkers by passage through a 10 ml sizing column before ethanol precipitation. This DNA fragment was then ligated in pBR322 previously isolated as missing the *Hind*III-to-*Bam*HI restriction fragment. *E. coli* strain RR1 was transformed to ampicillin in resistance using part of this ligation mix. After quick screen analysis of a number of recombinant plasmids, pJD221 which had the insert with the *Hind*III linker added to the end of the fragment closest to the ATG of the ADH structural gene was isolated by plasmid preparation.

pJD221 was linearized with *Hind*III and the resulting fragment then successively treated with exonoclease III and S_1 nuclease. The ends of these deleted plasmids were then made blunt using the Klenow fragment of DNA Polymerase I (see procedure above). After ethanol precipitation the ends of the DNA were ligated with *Xho*I linkers in a 12 hour reaction mixture. After digestion of resulting ligation mix with *Xho*I, plasmid solution was run in a 0.5 percent preparative agarose gel. DNA bands were cut from the gel, electroluted, then passed through a DE52 column before ethanol precipitation. Linear plasmid was circularized using T4 DNA ligase. The resulting ligation mix was used to transform *E. coli* strain RR1 to ampicillin resistance: All such colonies were pooled together. The resulting single plasmid pool was cut with *Xho*I and *Bam*HI, then run on a preparative 0.7 percent agarose gel. The 1500bp bands containing the ADH promoter region were cut from the gel, electroeluted then passed through a DE52 column before ethanol precipitation and ligation into the vector pY*ecyclΔx+1*. This plasmid had previously been isolated from an agarose gel as having lost the *Xho*I to BamHI restriction fragment described in the Figure. The resulting ligation was used to transform *E. coli* strain RRI to ampicillin resistance. Colonies were mixed for preparation of a plasmid pool which was then used to transform yeast strain GM-3C-2 to leucine prototrophy. Plasmids were then isolated from leucine prototrophs able to grow on glycerol plates. One plasmid, pACF 301, was found to contain a deletion extending toward the ATG of the ADH1 structural gene, leaving intact the first five triplets of the structural gene and the AC of the ACC of Thr_6 (Fig. 2b). This plasmid was digested with *Xho*I then treated with exonuclease *Bal*31 for 15 and 30 seconds (two different aliquots). Resulting plasmids were pooled, ethanol precipitated and then treated with DNA Polymerase I (reaction described above) so that all DNA ends were made blunt. *Eco*RI linkers were then added to the DNA solution and ligation allowed to proceed for 12 hours. After digestion with *Eco*RI and *Bam*HI, ligation mix was run on a preparative agarose gel. A DNA band about 1500 bp in size was cut from the gel, electroeluted then passed through a sizing column before ethanol precipitation. This DNA was then ligated into the linear pBR322 DNA previously isolated as missing the *Eco*RI-to-*Bam*HI restriction fragment. This ligation mix was used to transform *E. coli* strain 294 to ampicillin resistance. Plasmids isolated from these colonies are referred to as the pGBn plasmid series.

Miniscreen analysis of a number of different recombinant plasmids from the pGBn plasmid series indicated that nine particular plasmids had small *Bal* 31 generated deletions toward the ADH promoter region through the ATG of the ADH structural gene. All nine plasmids were digested with EcoRI, then end labeled by incubation with (α^{32}P)dATP and DNA polymerase I (conditions as described above). After ethanol precipitation, seven plasmids were digested with *Alu*I then electrophoresed on a 20 percent acrylamide—urea sequencing gel. ^{32}P-labelled plasmid DNAs from pGB904 and pGB906 were cut with *Bam*HI then run on a preparative gel. Labelled fragments containing the ADH promoter region were excised from the gel, electroluted, passed through a DE52 column before enthanol precipitation. These two resuspended fragments (from plasmids pGB904 and pGB906) were then subjected to the G+A and T+C sequence specific degradation reactions described by Maxam and Gilbert (procedure 11 and 12 respectively (23)). These sequencing reaction products were electrophoresed along with labeled fragments from pGB905, pGB914, pGB917, pGB919 and pGB921 on the thin 20 percent acrylamide sequencing gel

(described in the sequencing reference). Autoradiography was as described. This procedure allowed the determination of the extent of deletion of ADH promoter region as this region had previously been sequenced using all four Maxam-Gilbert sequencing reactions (J. Bennetzen, Ph.D Thesis, University of Washington, 1980).

Expression vector construction (Fig. 1): 10µg of YRp7 (24—26) was digested with *Eco*RI. Resulting sticky DNA ends were made blunt using DNA Polymerase I (Klenow fragment). Vector and insert were run on 1 percent agarose (SeaKem) gel, cut from the gel, electroeluted and 2X extracted with equal volumes of chloroform and phenol before ethanol precipitation. The resulting blunt end DNA molecules were then ligated together in a final volume of 50 µl for 12 hours at 12°C. This ligation mix was then used to transform *E. coli* strain JA300 to ampicillin resistance and tryptophan prototrophy. Plasmids containing the *TRP*1 gene in both orientations were isolated. pFRW1 had the *TRP*1 gene in the same orientation as YRp7 while pFRW2 had the *TRP*1 gene in the opposite orientation.

10 µg of pFRW1 and 10 µg of YRp7 were digested with *Hind*III then run in separate lanes on a 1 percent agarose gel. The large *Hind*III fragment from the pFRW1 lane and the small fragment from the YRp7 lane were eluted from the gel, extracted with phenol and chloroform, ethanol precipitated, then ligated for 12 hours at 15°C in a final volume of 50 µl. This ligate mix was used to transform JA300 to tryptophan prototrophy and ampicillin resistance. Plasmid (pFRL4) containing a single *Eco*RI site was then purified.

Referring now to Fig. 2, the pGBn plasmid series was digested with *Bam*HI and *Eco*RI then run on a 1 percent agarose gel. The ≈1500 bp promoter containing fragment from each lane was cut from the gel, electroeluted, then purified on a 10 ml diethylamino cellulose (Whatman) column before ethanol precipitation.

20 µg of pFRL4 was digested with *Bam*HI and *Eco*RI then run on a 1 percent agarose gel. The large (=5kb) fragment was cut from the gel, electroeluted, 2X extracted with phenol and chloroform before ethanol precipitation. 3 µg of this fragment was then separately ligated with each of the promoter containing fragments for 12 hours at 15°C in 50 µl ligation mix. *E. coli* K-12 strain 294 was transformed with the ligation mix to ampicillin resistance and plasmids from each of these different transformation mixtures were purified (pFRPn plasmid genes).

10 µg of pLeIF D (5) was digested with *Eco*RI then run on a 6 percent acrylamide gel. The 560 bp leukocyte interferon D gene was cut from the gel, electroeluted and 2X extracted with phenol/chloroform before ethanol precipitation. This interferon gene was then ligated into the unique *Eco*RI site in the pFRPn plasmids previously cut with *Eco*RI and treated with bacterial alkaline phosphatase. These vectors were then used for *Bgl*II restriction analysis and yeast transformations.

Interferon assay: Extracts of yeast were assayed for interferon by comparison with interferon standards by the cytopathic effect (CPE) inhibition assay (27). Yeast extracts were prepared as follows: Five ml cultures were grown in YNB+CAA until reaching A_{650}=1-2. Cells were collected by centrifugation then resuspended in 600 µl of 1.2 M sorbitol, 10 mM KH_2PO_4, pH=6.8 and 1 percent zymolyase 60,000 then incubated at 30°C for 30 min. Spheroplasts were pelleted at 3000 xg for 10 min., then resuspended in 150 µl of 7 M guanidine hydrochloride plus 1mM phenylmethylsulfonylfluoride (PMSF). Extracts were diluted 1,000 fold in PBS buffer (20 mM NaH_2PO_4, pH=7.4, 150 mM NaCl, 0.5 percent BSA) immediately before the assay.

Results

Construction of a vector for insertion of a series of promoter fragments and for insertion of a gene to be expressed

To design a plasmid vector for autonomous replication in yeast, it is necessary to have both an origin of replication and a gene present for selection in yeast. Furthermore, the plasmid must contain a bacterial plasmid origin of replication and a means of selection in bacteria (e.g., an antibiotic resistance gene). With these requirements a plasmid can be constructed and modified *in vitro* using recombinant DNA techniques, amplified in bacteria, preferably *E. coli*, and finally transformed into yeast.

Such a vector is shown in Fig. 1 and is designated YRp7 (24—26). It contains a chromosomal origin of replication from yeast (*ars*1) as well as the *TRP1* gene which codes for N-(5'-phosphoribosyl)-anthranilate isomerase (28). The *TRP1* yeast gene can complement (allow for growth in the absence of tryptophan) *trp1* mutations in yeast (e.g., RH218, see Methods) and can also complement the *trpC*1117 mutation of *E. coli* (e.g. JA300) (18). The plasmid is pBR322 (29) based so as it also permits growth and selection in *E. coli* using antibiotic resistance selection.

Since it was necessary to clone into this vector *Bam*HI/*Eco*RI restriction fragments containing a yeast promoter, it proved convenient to first remove one *Eco*RI site from the vector. This was done as shown in Fig. 1. The vector YRp7 was cut was *Eco*RI followed by filling in of the sticky *Eco*RI ends of both fragments with Klenow DNA polymerase I. The fragments were then blunt end ligated and the resulting DNA was used to transform *E. coli* JA300 to Trp^+ and ampicillin resistance (Ap^R). In such a way plasmid pFRW1 was isolated with both *Eco*RI sites removed.

One *Eco*RI site was then restored to the plasmid in order than an *Eco*RI/*Bam*HI fragment could be later cloned into the vector. This was done by cutting both YRp7 and pFRW1 with *Hind*III followed by the isolation of the fragments indicated. When the small *Hind*III fragment of YRp7 was put together with the

large *Hind*III fragment of pFRW1, pFRL4 was obtained. It was selected for in *E. coli* JA300 using Trp$^+$ and Ap^R phenotypes.

Construction of yeast alcohol dehydrogenase (ADH) promoter fragments

Since it is not known whether certain specific sequences in the leader region preceding structural genes are required for RNA polymerase II binding or what DNA is necessary for ribosome recognition (ribosome binding sites) of the mRNA, promoter fragments from the ADH gene (*ADC1*) were obtained as described in Fig. 2.

The first step was to show that the 5'-leader DNA sequence of the ADH gene could be used to express another structural gene from yeast without its leader sequence (*CYC1*). Thus a plasmid which can complement a *cyc*1 mutation in yeast can be used to isolate the ADH promoter fragment that will result in *cyc*1 expression. This promoter fragment could then be used to express other eukaryotic genes (e.g., the Leukocyte Interferon D gene).

As shown in Fig. 2, pY9T6 containing the *ADC*1 locus (Bennetzen, supra) was cut with *Sau*3A to isolate the 5'flanking sequence of the ADH gene on an approximately 1600 bp fragment. The ATG translation start for the ADH coding sequence is shown with the A at position +1, and transcription goes from left to right as shown. This fragment was blunt ended using Klenow DNA polymerase I followed by a ligation with a mixture of *Bam*HI and *Hind*III linkers. After cutting with *Bam*HI and *Hind*III, the fragments were ligated with the large *Bam*HI/*Hind*III fragment of pBR322. The ligation products were used to transform *E. coli* to Ap^R and the desired pJD221 was isolated from a transformant colony using a standard miniscreen procedure (see Methods). pJD221 was cut with *Hind*III and then with exonuclease III and S_1 nuclease to remove base pairs toward but not through the ATG of the ADH structural gene.

This procedure also removes base pairs in the opposite direction (toward the *Eco*RI site) at approximately the same rate. The reaction was designed so as to not remove the ATG of ADH since the ATG of *CYC*1 was not present in the fragment to be expressed under ADH promoter control. Therefore a complementation of *cyc*1 yeast would require a functional ADH1—CYC1 fusion protein.

The end-deleted products were treated with Klenow DNA polymerase I to ensure blunt ends followed by the addition of *Xho*I linkers by blunt end ligation. After *Xho*I cutting a circular plasmid was regenerated by ligation. Plasmids containing gel-determined, properly sized *Eco*RI-to-*Bam*HI restriction fragments were digested with *Xho*I, then ligated with the large *Xho*I-to-*Bam*HI restriction fragment of plasmid pYe*cyc 1*Δx+1. After amplification in *E. coli* RR1 resulting plasmids were used to transform a *cyc1 cyc3 leu1* yeast strain to leucine prototrophy on minimal glucose plates. Growing colonies were patched onto glycerol/ethanol plates. Yeast able to grow on such plates require the presence of functional cytochrome-c protein. This can only occur on this plasmid if fragments containing ADH promoter deletions can initiate (in the correct reading frame) translation of the cytochrome c-coding region. Plasmid pACF301 was isolated from one such transformant. The junction between ADH1 and CYC1 is shown at the bottom of Fig. 2b. Six amino acid codons from the ADH sequence were present with 3 new amino acid codons due to the *Xho*I linker, and the rest represented the *CYC*1 structural gene. Thus the ADH promoter fragment is expressing a fusion gene product that produces a phenotypically active CYC1 gene fusion product.

In the construction of a yeast expression plasmid, it is desirable that the ATG codon of the non-yeast gene to be expressed be the one belonging to the same non-yeast gene rather than a vector ATG which would lead to the synthesis of an undesired fusion protein. Therefore, it proved appropriate to remove nucleotides through the ATG of the ADH promoter fragment by another series of deletions and supply a new translation start signal with the gene to be expressed. Since the functionality of upstream DNA sequence (−1 to −1500) during the expression process is not known, it was desirable to remove as little sequence as possible upstream from the ATG and to try different fragments lacking both the initially present ATG and various amounts of additional DNA sequence.

These additional promoter fragments were isolated as shown in Fig. 2b. pACF301 was cut with *Xho*I and *Bal*31. After blunt-ending, addition of *Eco*RI linker, *Bam*HI/*Eco*RI cutting, and sizing fragments; the correct size class of fragments were ligated with *Eco*RI/*Bam*HI-cut pBR322. Specific recloned ADH promoter fragments were isolated from plasmids from various *E. coli* Ap^R transformants.

Fig. 3 shows the DNA sequences of the transcribed strand of 8 of the resulting, variously sized and numbered promoter fragments. The numbered lines show where the right end of the fragment ends and where the *Eco*RI linker sequence begins.

The ends of fragments 904 and 906 were exactly determined by sequencing. The *Eco*RI sticky ends of these fragments were labelled with Klenow DNA polymerase using α-^{32}P-dATP. A sequencing gel was used to read from the A's into the linker through the junction. The other 6 fragment ends were approximated to within about 1—2 base pairs by labelling as above, cutting with *Alu*I, followed by sizing on the same denaturing gel.

Construction of plasmids that express biologically active leukocyte interferon D in yeast

In order to optimize for successful expression of LeIF D in yeast, eight different promoter fragments (Fig. 3) were ligated into the pFRL4 vector as shown in Fig. 4.

The vector was designed to have ADH promoter transcription in the same direction as *TRP*1 gene transcription (31). Since the LeIF D gene was to be inserted in the *Eco*RI site and was not known to contain

proper 3' termination and processing sequences for yeast recognition. The *TRP*1 gene flanking sequence was aligned to perform these functions.

The resulting pFRPn series (where n is the promoter fragment number) was obtained as shown. The preferred embodiment of these, pFRP6 in a transformant strain of *E. coli* 294, has been deposited in the American Type Culture Collection (ATCC No. 31814, deposited 24 Feb. 1981). These vectors were cut with *Eco*RI, alkaline phosphatase treated (to avoid premature recircularisation), and ligated with the *Eco*RI LeIF D gene fragment. The ATG of this gene immediately follows the C of the *Eco*RI linker (GAATTC*ATG*) as shown (Fig. 4).

Ampicillin resistant transformants of *E. coli* K-12 strain 294 were screened to find plasmids containing both orientations of the LeIF D fragment (pFRSn series—n refers to screening number). Orientations were determined by agarose gel electrophoresis using *Bgl*II digestion which cuts both in the vector and in the LeIF D gene as shown.

Three of the plasmids demonstrated unpredicted restriction patterns. pFRS7 and pFRS35 have an extra *Bgl*II fragment at 560 bp. This results from having two fragments of LeIF D in line with ADH transcription. pFRS16 has no proper orientation fragment but has a 1700 bp fragment which apparently resulted from the ligation of the two vector fragments together (two *TRP*1 containing "tails" together) with one LeIF D fragment in between two "heads" containing ADH promoter fragments. Thus in this ligation product the interferon gene is in the proper orientation for expression by one of the ADH promoter fragments.

Evidence for leukocyte interferon D expression in yeast

First the *E. coli* 294 strains containing the various plasmids (pFRSn) were grown and extracts prepared (see Methods). No interferon activity was observed in the extracts using the cytopathic effect inhibition assay (see Methods). However, when plasmids with purified and used for yeast transformations by selection of TRP^+ phenotype using yeast RH218 (*trp*1 mutation), all plasmids with orientation I produced interferon activity in yeast while no plasmids with orientation II produced interferon.

Table 1 shows the results of interferon assays which measure antiviral activity effects on VSV virus challenge of MDBK tissue culture cells (see Methods). Seven of the promoter fragments definitely express the LeIF D gene when the gene is in the proper orientation (I). This is demonstrated by comparing units/(ml of extract) for the orientation I plasmids with the orientation II plasmids. All orientation II plasmids expressed <1900 units/(ml of extract), a value 1 to 4 percent of the values for orientation I plasmids (actually background values are probably much lower than this since the 1900 value is a function of the assay procedure).

TABLE 1
Interferon activity in yeast extracts

Orientation	ADH promoter fragment	Plasmid	Units[a]/ml of extract	Units/l of cells $\times 10^{-6}$	Units[b]/l of cells at Abs=1 $\times 10^{-6}$	cells[c]/l of culture $\times 10^{-10}$	Pct.[c] of cells with plasmid	Units/ cell containing plasmid $\times 10^{4}$	Molecules[d]/ cell containing plasmid
I	904	pFRS3	47,000	1.4	1.2	2.1	19	3.5	53,000
I	905	pFRS7	47,000	1.4	0.54	3.4	30	1.4	21,000
I	913	pFRS16	187,500	5.6	2.0	5.1	32	3.4	51,000
I	906	pFRS12	125,000	3.8	1.8	2.7	19	7.4	110,000
I	906	pFRS36	187,500	5.6	2.3	3.0	16	12	180,000
I	915	pFRS23	125,000	2.1	0.81	3.6	21	2.8	42,000
I	921	pFRS35	250,000	7.5	2.8	4.5	18	9.2	140,000
I	919	pFRS34	93,750	2.8	1.4	2.2	17	7.6	110,000
II	904	pFRS2	<1900				22		
II	905	pFRS6	"				23		
II	913	pFRS17	"				36		
II	906	pFRS11	"				13		
II	915	pFRS22	"				46		
II	921	pFRS26	"				25		
II	919	pFRS33	"				31		

[a] Yeast cells were grown in 5 ml of YNB+CAA (Trp^+ selection) to an absorbance (Abs) of 1.2 to 2.8 at 660 mμ and spheroplasted with zymolyase as described in Methods. The final pellet was resuspended in 0.15 ml of 7 M guanidine HCl and 1 mM PMSF. The extracts were assayed using 1/100 or 1/1000 dilutions in dilution buffer using an VSV virus challenge of MDBK cells (see Methods).

[b] Units/liter of cells at $Abs_{660}=1\times10^{-5}$ is a normalization since different cultures were stopped between 1.2 to 2.8 Abs_{660}.

[c] The number of yeast cells per culture was determined by dilution and plating on YNB+CAA+tryptophan (50 μg/ml) plates. To find out how many cells have plasmid · (Trp^+ complementation) cultures were also plated on YNB+CAA (without tryptophan). Thus percentage of cells containing plasmid was determined.

[d] Molecules/(cell containing plasmid) was calculated assuming purified leukocyte interferon D has a specific activity of 2×10^8 units/mg (27) in the same assay and a molecular weight of 20,000 g/mole.

Since *ars*1 (chromosomal) origin of replication)-containing plasmids have previously been shown to be unstable and lost in a high percentage of the cells even under selective maintenance pressure (24, 25), the percent of cells containing the plasmid at the time of extract preparation was measured. This was done by plating diluted cultures on plates with and without tryptophan. The results of this demonstrate that the plasmid is somewhat unstable in yeast (though not in bacteria), but can be maintained by growth under selective pressure. These results also are evidence for the presence of the plasmid, since RH218 (*trp*1) yeast do not grow on plates without tryptophan and since a revertant to TRP^+ would plate with equal efficiency on plates with and without tryptophan. Furthermore, the percentages of cells containing plasmid are similar comparing yeast with orientation I and II plasmids. This suggests that the production of interferon in the yeast cell does not result in increased instability of the plasmid due to interferon toxicity to the cell.

The fact that all the promoter fragments express interferon when up to 32 bp are removed upstream from the ATG suggests that the DNA sequence in this region is relatively unimportant in transcription and translation. These results also suggest that precise spacing between the promoter and the ATG may be relatively unimportant for expression in yeast.

In addition, Table 1 shows molecules/cell values which are very much higher than the 10,000 molecules/cell observed for interferon D expression in *E. coli* on a high copy plasmid with a strong promoter (*trp* promoter) (32). Assessment of this extreme difference (up to 18 fold) in molecules per cell should recognize that the yeast cell volume is probably 2 orders of magnitude higher than that of *E. coli*; however, the amount of expression from only 1—2 copies of the yeast plasmid versus the high copy number of plasmids producing interferon in *E. coli* is dramatic.

Comparison of the size of interferon produced in yeast versus *E. coli*

Since the interferon gene uses its own ATG-initiation codon and since the alcohol dehydrogenase ATG has been removed in the construction, one would expect to find that the interferon expressed in yeast is the same size as the interferon in *E. coli* (32). SDS-polyacrylamide gel electropheresis was accordingly done on a *E. coli* extract containing interferon D versus a yeast extract containing interferon D. After running the gel, two lanes containing yeast extract versus E. coli extracts were simultaneously sliced. The slices were put into assay dilution buffer and left at 4°C for 3 days. Interferon assays were then performed to compare sizes of the peptides. Both appear to be about 20,000 daltons, the size expected for interferon D. However, there does appear to be a slight difference in the molecular weights, with yeast interferon D being about 7 percent larger, possibly owing to glycosylation. Despite the size difference, the products of yeast expression exhibited interferon activity (Table 1).

The preceding data clearly demonstrates that a yeast 5'-flanking DNA sequence, without the translation start signal of the structural gene, can efficiently promote the expression of an inserted mammalian or other structural gene for a biocompetent polypeptide, and do so without the aid of selective pressure for the product of expression (i.e., the expression product is not required for cell growth).

The availability of yeast promoter-containing plasmids (pFRPn series) having both yeast and bacterial phenotypical genes and origins of replication, and a site downstream from the promoter convenient for the insertion of translation start- and stop-bearing structural genes permits the creation of DNA expression vectors for a wide variety of polypeptides. Thus, into such a site may be inserted, for example, structural genes for both normal (5) and hybrid (6) human leukocyte interferons, fibroblast interferon (7), somatostatin or the A or B chains of human insulin (1), human proinsulin (2), thymosin alpha 1 (3), human growth hormone (4) and, indeed, virtually any other biocompetent polypeptide.

Following expression, product may be extracted and purified as in the case of bacterial expression, *mutatis mutandis*

It will be appreciated that the invention is not limited in its application to the particular expression vector exemplified above. For example, use of the so-called two micron origin of replication would provide additional stability, making unnecessary resort to selective pressure for maintenance of the plasmid in the yeast cell, particularly if the host strain is [CIR+], i.e., contains normal two micron plasmid (33). Such an expression vector would be stable in yeast in the rich medium ordinarily best for large scale fermentations. At the same time, use of the two micron origin of replication could significantly increase plasmid copy number in each cell.

Stability of the expression vector in yeast may also be enhanced by inclusion within the plasmid of a yeast centromere (34), an element involved in maintenance of the yeast chromosome. The resulting plasmid will behave as a minichromosome, such that selective pressure will not be required during growth or maintenance of the plasmid. As many as 17 different yeast centromeres have been identified to the present date.

Transcription terminators other than that present on the *TRP1* gene may be employed, e.g., other 3'-flanking sequences from yeast such as the 3'-flanking sequence contained on a *Hind* II-*Bam*HI fragment of the ADH 1 gene.

Optimization may also result from alteration of the sequence between the yeast promoter fragment and the inserted gene fragment. Thus, an A (adenine base) is found at position −3 (the third base before the translation start signal) of all twenty different mRNA-coding yeast genes heretofore sequenced. A variety of means (e.g., use of linkers) for including such an element in the plasmids of the invention will appear to those skilled in the art.

Of course, promoters other than the ADH promoter exemplified above may be employed in variants of the invention. For example, the promoter of the yeast 3-phosphoglycerate kinase gene may be employed, doubtless increasing expression levels significantly over those observed for the ADH system. Again, one or more of the promoters for yeast glyceraldehyde-3-phosphate dehydrogenase may be employed. This system is nonfunctional in the absence of glucose, but induced 200-fold in its presence, and could accordingly be employed for fine control of expression.

From the foregoing, it will be apparent that the invention provides new means for the expression of valuable polypeptides. In particular instances, efficiency of expression relative to that in recombinant bacteria may result from the different codon usage patterns as between yeast and bacteria, such that eukaryotic genes may be better expressed in yeast. The yeast expression systems of the invention may also provide advantage in the glycosylation of biocompetent polypeptides, an ability bacteria lack. The glycosylation system of yeast is very similar to that of higher eukaryotes, and glycosylation may prove to have profound effects on the functions of proteins.

As will be apparent to those skilled in the art in the light of the foregoing discussion, the invention is not to be limited to the preferred embodiments thereof exemplified above.

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Claims for the Contracting States: BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. A DNA vector suitable for use in expressing exogenous genes in yeast, comprising a sequence which is replicable in yeast, a 5' flanking sequence of a yeast structural gene including a promoter, a site downstream of said 5' flanking sequence in the direction of transcription for insertion of a structural gene coding for a polypeptide ordinarily exogenous to yeast so as to be transcribable under the control of said promoter and translatable from a start signal, and a sequence allowing phenotypic selection of yeast transformants.

2. A DNA vector according to claim 1 which additionally comprises a transcription termination sequence downstream of said insertion site.

3. A DNA vector according to claim 2 wherein said transcription termination sequence is a flanking sequence of a yeast gene.

4. A DNA vector according to claim 3 wherein said yeast gene provides the phenotypic selection of yeast transformants.

5. A DNA vector according to any one of the preceding claims wherein said 5' flanking sequence lacks a translation start signal upstream of said insertion site with respect to the direction of translation.

6. A DNA vector according to any one of the preceding claims wherein said 5' flanking sequence lacks the translation start signal of the gene from which it is derived.

7. A DNA vector according to claim 6 wherein said insertion site lies at or upstream, with respect to the direction of translation, of the position normally occupied by the translation start signal of the gene from which it is derived.

8. A DNA vector according to any one of the preceding claims wherein said 5' flanking sequence is that of the ADH1 structural gene.

9. A DNA vector according to any one of the preceding claims wherein said sequence for phenotypic selection in yeast is the TRP1 gene.

10. A DNA vector according to any one of the preceding claims which additionally comprises a bacterial origin of replication and one or more sequences for phenotypic selection in bacteria.

11. A recombinant DNA vector for use in expressing an exogenous structural gene in a suitable yeast strain, comprising a DNA vector according to any one of the preceding claims and a said exogenous gene inserted at said site so as to be transcribable under the control of said promoter and translatable from a start signal.

12. A recombinant DNA vector according to claim 11 wherein said exogenous gene encodes a biocompetent polypeptide.

13. A recombinant DNA vector according to claim 11 wherein said exogenous gene encodes a mammalian polypeptide.

14. A recombinant DNA vector according to claim 13 wherein said mammalian polypeptide is selected from normal and hybrid human interferons, human proinsulin, the A and B chains of human insulin, human growth hormone, somatostatin and thymosin alpha 1.

15. A recombinant DNA vector according to claim 13 wherein said mammalian polypeptide is that of leukocyte interferon D.

16. A yeast strain transformed with a recombinant DNA vector according to any one of claims 11 to 15 capable of expressing said exogenous gene to produce a polypeptide.

17. A transformed yeast strain according to claim 16 wherein said polypeptide is not required for the growth of the transformant.

18. A transformed yeast strain according to claim 16 or claim 17 wherein in said recombinant DNA vector the phenotypic gene for selection in yeast complements a mutation carried by the yeast strain.

19. A transformed yeast strain according to any one of claims 16, 17 and 18 wherein the yeast is of the genus Saccharomyces.

20. A transformed yeast strain according to claim 19 wherein the yeast is of the species Saccharomyces cerevisiae.

21. A transformed yeast strain according to claim 20 wherein the yeast is the strain Saccharomyces cerevisiae RH 218.

22. A method of forming a transformed yeast strain of any one of claims 16 to 21, which method includes:

(a) providing a DNA transfer vector replicable in both bacteria and yeast and genes for phenotypic selection of both bacterial and yeast transformants;

(b) providing a DNA fragment comprising a structural gene encoding a polypeptide ordinarily exogenous to yeast;

(c) providing a DNA fragment comprising a yeast promoter genetically distinct from said exogenous structural gene;

(d) inserting the fragments of (b) and (c) into said transfer vector with appropriately positioned translation start and stop signals for said exogenous gene to form a recombinant DNA vector in which transcription of said exogenous gene is under the control of said promoter and translation is from said start signal, the bacterial replication and phenotypic selection being employed for amplification of the DNA in bacteria at intermediate stages in the vector construction; and

(e) transforming a suitable yeast strain with the resulting vector so that the exogenous gene is expressible in the transformant.

23. A method of producing a heterologous polypeptide which comprises culturing a yeast strain of any one of claims 16 to 22, and isolating said biocompetent polypeptide from the culture medium.

24. A method of producing a desired heterologous polypeptide in yeast by culturing a yeast strain transformed with a recombinant DNA expression vector replicable in said yeast strain, characterised in that the vector contains an exogenous DNA sequence coding for the polypeptide transcriptionally downstream of a 5' flanking sequence of a yeast structural gene containing a promoter which is functional in said yeast strain, and a translation initiation signal between said promoter and the exogenous coding sequence, so that the exogenous sequence is transcribed from said promoter and translated from said translation initiation signal.

25. A method according to claim 24 wherein the expression vector additionally includes a transcription termination sequence downstream of said exogenous coding sequence.

26. A method according to claim 24 or claim 25 wherein the translation initiation signal lies at or upstream of the position normally occupied by the translation initiation signal of the yeast structural gene from which said 5' flanking sequence is derived.

Claims for the Contracting State: AT

1. A method of forming a DNA vector suitable for use in expressing exogenous genes in yeast, which method includes ligating DNA fragments together to form a vector comprising a sequence which is replicable in yeast, a 5' flanking sequence of a yeast structural gene including a promoter, a site downstream of said 5' flanking sequence in the direction of transcription for insertion of a structural gene coding for a polypeptide ordinarily exogenous to yeast so as to be transcribable under the control of said promoter and translatable from a start signal, and a sequence allowing phenotypic selection of yeast transformants.

2. A method according to claim 1 which includes providing a transcription termination sequence downstream of said insertion site.

3. A method according to claim 2 wherein said transcription termination sequence is a flanking sequence of a yeast gene.

4. A method according to claim 3 wherein said yeast gene provides the phenotypic selection of yeast transformants.

5. A method according to any one of the preceding claims wherein said 5' flanking sequence lacks a translation start signal upstream of said insertion site with respect to the direction of translation.

6. A method according to any one of the preceding claims wherein said 5' flanking sequence lacks the translation start signal of the gene from which it is derived.

7. A method according to claim 6 wherein said insertion site lies at or upstream, with respect to the direction of translation, of the position normally occupied by the translation start signal of the gene from which it is derived.

8. A method according to any one of the preceding claims wherein said 5' flanking sequence is that of the ADH1 structural gene.

9. A method according to any one of the preceding claims wherein said sequence for phenotypic selection in yeast is the TRP1 gene.

10. A method according to any one of the preceding claims which includes providing a bacterial origin of replication and one or more sequences for phenotypic selection in bacteria, and using the same for amplification of DNA in bacteria at intermediate stages in the vector construction.

11. A method according to any one of the preceding claims which comprises additionally ligating in the vector at said insertion site a said exogenous structural gene coding for a bicompetent polypeptide so as to be transcribable under the control of said promoter and translatable from a start signal.

12. A method according to claim 11 wherein said exogenous gene encodes a biocompetent polypeptide.

13. A method according to claim 11 wherein said exogenous gene encodes a mammalian polypeptide.

14. A method according to claim 13 wherein said mammalian polypeptide is selected from normal and hybrid human interferons, human proinsulin, the A and B chains of human insulin, human grown hormone, somatostatin and thymosin alpha 1.

15. A method according to claim 13 wherein said mammalian polypeptide is that of leukocyte interferon D.

16. A method of forming a yeast transformant capable of expressing an exogenous gene to produce a polypeptide which comprises transforming a suitable yeast strain with a recombinant DNA vector according to any one of claims 11 to 15.

17. A method according to claim 16 wherein said polypeptide is not required for the growth of the transformant.

18. A method according to claim 16 or claim 17 wherein in said recombinant DNA vector the phenotypic gene for selection in yeast complements a mutation carried by the yeast strain.

19. A method according to any one of claims 16, 17 and 18 wherein the yeast is of the genus Saccharomyces.

20. A method according to claim 19 wherein the yeast is of the species Saccharomyces cerevisiae.

21. A method according to claim 20 wherein the yeast is the strain Saccharomyces cerevisiae RH 218.

22. A method of producing a heterologous polypeptide which comprises culturing a yeast strain of any one of claims 16 to 21, and isolating said biocompetent polypeptide from the culture medium.

23. A method of producing a desired heterologous polypeptide in yeast by culturing a yeast strain transformed with a recombinant DNA expression vector replicable in said yeast strain, characterised in that the vector contains an exogenous DNA sequence coding for the polypeptide transcriptionally downstream of a 5' flanking sequence of a yeast structural gene containing a promoter which is functional in said yeast strain, and a translation initiation signal between said promoter and the exogenous coding sequence, so that the exogenous sequence is transcribed from said promoter and translated from said translation initiation signal.

24. A method according to claim 23 wherein the expression vector includes a transcription termination sequence downstream of said exogenous coding sequence.

25. A method according to claim 23 or claim 24 wherein the translation initiation signal lies at or upstream of the position normally occupied by the translation initiation signal of the yeast structural gene from which said 5' flanking sequence is derived.

Patentansprüche für die Vertragsstaaten: BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Ein DNS-Vektor, der für die Expression von exogenen Genen in Hefe geeignet ist, umfassend eine Sequenz, die in Hefe replizierbar ist, eine 5'-Flankensequenz eines Hefestrukturgens, das einen Promotor umfaßt, eine Stelle stromabwärts von der genannten 5'-Flankensequenz in Richtung der Transkription zur Einfügung einer Strukturgenkodierung für ein Polypeptid, das normalerweise exogen zu Hefe ist, sodaß es unter der Steuerung des genannten Promotors transkribierbar und von einem Startsignal translatierbar ist, und eine Sequenz, die die phenotypische Selektion von Hefetransformanten zuläßt.

2. Ein DNS-Vektor nach Anspruch 1, der zusätzlich eine Transkriptionsterminationssequenz stromabwärts von der genannten Einfügungsstelle aufweist.

3. Ein DNS-Vektor nach Anspruch 2, worin die genannte Transkriptionsterminationssequenz eine Flankierungssequenz eines Hefegens ist.

4. Ein DNS-Vektor nach Anspruch 3, worin das genannte Hefegen die Phenotypische Selektion von Hefetransformanten schafft.

5. Ein DNS-Vektor nach einem der vorhergehenden Ansprüche, worin der genannten 5'-Flankensequenz ein Translationsstartsignal stromaufwärts von der genannten Einfügungsstelle in bezug auf die Translationsrichtung fehlt.

6. Ein DNS-Vektor nach einem der vorhergehenden Ansprüche, worin der genannten 5'-Flankensequenz das Gens fehlt, aus dem sie abgeleitet ist.

7. Ein DNS-Vektor nach Anspruch 6, worin die genannte Einfügungsstelle in bezug auf die Translationsrichtung an der oder stromaufwärts von der Stelle gelegen ist, die normalerweise von dem Translationsstartsignal des Gens eingenommen wird, von dem sie abgeleitet ist.

8. Ein DNS-Vektor nach einem der vorhergehenden Ansprüche, worin die genannte 5'-Flankensequenz jene des AHD1-Strukturgens ist.

9. Ein DNS-Vektor nach einem der vorhergehenden Ansprüche, worin die genannte Sequenz für die phenotypische Selektion in Hefe das TRP1-Gen ist.

10. Ein DNS-Vektor nach einem der vorhergehenden Ansprüche, das zusätzlich einen bakteriellen Replikationsursprung und eine oder mehrere Sequenz(en) für die phenotypische Selektion in Bakterien umfaßt.

11. Ein rekombinanter DNS-VEktor für die Verwendung zur Expression eines exogenen Strukturgens in einem geeigneten Hefestamm, umfassend einen DNS-Vektor nach einem der vorhergehenden Ansprüche

und ein genanntes exogenes Gen, das an der genannten Stelle eingefügt wird, um unter der Steuerung des genannten Promotors transkribierbar und von einem Startsignal translatierbar zu sein.

12. Ein rekombinanter DNS-Vektor nach Anspruch 11, worin das genannte exogene Gen ein biokompetentes Polypeptid kodiert.

13. Ein rekombinanter DNS-Vektor nach Anspruch 11, worin das genannte exogene Gen ein Säugetier-Polypeptid kodiert.

14. Ein rekombinanter DNS-Vektor nach Anspruch 13, worin das genannte Säugetier-Polypeptid aus normalen und hybriden menschlichen Interferonen, menschlichem Proinsulin, den A- und B-Ketten von menschlichem Insulin, menschlichem Wachstumshormon, Somatostatin und Thymos in Alpha 1 gewählt wird.

15. Ein rekombinanter DNS-Vektor nach Anspruch 13, worin das genannte Säugetier-Polypeptide das von Leukocyt-Interferon D ist.

16. Ein Hefestamm, der mit einem rekombinanten DNS-Vektor nach einem der Ansprüche 11—15 trnsformiert ist, der fähig ist, das genannte exogene Gen zu exprimieren, um ein Polypeptid zu erzeugen.

17. Ein transformierter Hefestamm nach Anspruch 16, worin das genannte Polypeptid für das Wachstum des Transformaten nicht erforderlich ist.

18. Ein transformierter Hefestamm nach Anspruch 16 oder 17, worin in dem genannten rekombinanten DNS-Vektor das phenotypische Gen für die Selektion in Hefe eine Mutation komplementiert, die vom Hefestamm getragen wird.

19. Ein transformierter Hefestamm nach einem der Ansprüche 16, 17 und 18, worin die Hefe zur Gattung Saccharomyces gehört.

20. Ein transformierter Hefestamm nach Anspruch 19, worin die Hefe zur Spezies Saccharomyces cerevisiae gehört.

21. Ein transformierter Hefestamm nach Anspruch 20, worin die Hefe der Stamm Saccharomyces cerevisiae RH 218 ist.

22. Ein Verfahren zur Bildung eines transformierten Hefestammes nach einem der Ansprüche 16—21, welches Verfahren umfaßt:

a) Schaffung eines DNS-Transfervektors, der sowohl in Bakterien als auch Hefe replizierbar ist und von Genen für die phenotypische Selektion von sowohl Bakterien—als auch Hefetransformanten;

b) Schaffung eines DNS-Fragmentes, das ein Strukturgen umfaßt, das ein Polypeptid kodiert, das normalerweise exogen zu Hefe ist;

c) Schaffung eines DNS-Fragmentes, das einen Hefepromotor umfaßt, der sich genetisch von dem genannten exogenen Strukturgen unterscheidet;

d) Einfügen der Fragmente aus b) und c) in den genannten Transfervektor mit in geeigneter Stellung vorgesehenen Translationsstart- und Haltesignalen für das genannte exogene Gen, um einem rekombinanten DNS-Vektor zu schaffen, in dem die Transkription des genannten exogenen Gens unter der Steuerung des genannten Promotors vor sich geht und die Translation vom genannten Startsignal aus vor sich geht, wobei die bakterielle Replikation und phenotypische Selektion für die Verstärkung der DNS in Bakterien in Zwischenstadien im Vektoraufbau verwendet wird; und

e) Transformation eines geeigneten Hefestammen mit dem entstandenen Vektor, sodaß das exogene Gen im Transformanten exprimierbar ist.

23. Ein Verfahren zur Herstellung eines heterologen Polypeptides, umfassend das Kultivieren eines Hefestammes nach einem der Ansprüche 16—22, und das Isolieren des genannten biokompetenten Polypeptides aus dem Kulturmedium.

24. Ein Verfahren zur Herstellung eines gewünschten heterologen Polypeptides in Hefe durch Kultivieren eines Hefestammes, der mit einem rekombinanten DNS-Expressionsvektor transformiert ist, der in dem genannten Hefestamm replizierbar ist, dadurch gekennzeichnet, daß der Vektor eine exogene DNS-Sequenzkodierung für das Polypeptid transkriptionell stromabwärts von einer 5'-Flankensequenz eines Hefestrukturgens enthält, das einen Promotor enthält, der in dem genannten Hefestamm funktionell ist, und ein Translationsauslösungssignal zwischen dem genannten Promotor und der exogenen Kodierungssequenz vorgesehen ist, sodaß die exogene Sequenz vom genannten Promotor transkribiert und vom genannten Translationsauslösungssignal translatiert wird.

25. Ein Verfahren nach Anspruch 24, worin der Expressionsvektor zusätzlich eine Transkriptionsterminationssequenz stromabwärts von der genannten exogenen Kodierungssequenz umfaßt.

26. Ein Verfahren nach Anspruch 24 oder 25, worin das Translationsauslösungssignal an der oder stromaufwärts von der Stelle gelegen ist, die normalerweise vom Translationsauslösungssignal des Hefestrukturgens eingenommen wird, von dem die genannte 5'-Flankensequenz abgeleitet ist.

Patentansprüche für den Vertragsstaat: AT

1. Ein Verfahren zur Bildung eines DNS-Vektors, der zur Verwendung bei der Expression von exogenen Genen in Hefe geeignet ist, welches Verfahren das Legieren von DNS-Fragmenten aneinander, um einen Vektor zu bilden, der eine Sequenz umfaßt, die in hefe replizierbar ist, eine 5'-Flankensequenz eines Hefestrukturgens umfassend einen Promotor, eine Stelle stromabwärts von der genannten 5'-Flankensequenz in Transkriptionsrichtung zum Einfügen einer Strukturgenkodierung für ein Polypeptid,

das normalerweise exogen zu Hefe ist, sodaß es unter der Steuerung des genannten Promotors transkribierbar und von einem Startsignal translatierbar ist, und einen Sequenz umfaßt, die die phenotypische Selektion von Hefetransformanten zuläßt.

2. Ein Verfahren nach Anspruch 1, das das Vorsehen einer Transkriptionsterminationssequenz stromabwärts von der genannten Einfügungsstelle umfaßt.

3. Ein Verfahren nach Anspruch 2, worin die genannte Transkriptionsterminationssequenz eine Flankensequenz des Hefegens ist.

4. Ein Verfahren nach Anspruch 3, worin das genannte Hefegen die phenotypische Selektion von Hefetransformanten bewirkt.

5. Ein Verfahren nach einem der vorhergehenden Ansprüche, worin der genannten 5'-Flankensequenz ein Translationsstartsignal stromaufwärts von der genannten Einfügungsstelle in bezug auf die Translationsrichtung fehlt.

6. Ein Verfahren nach einem der vorhergehenden Ansprüche, worin der genannten 5'-Flankensequenz das Translationsstartsignal des Gens fehlt, von dem sie abgeleitet ist.

7. Ein Verfahren nach Anspruch 6, worin die genannte Einfügungsstelle in bezug auf die Translationsrichtung an der oder stromaufwärts von der Stelle liegt, die normalerweise vom Translationsstartsignal des Gens aufgenommen wird, von dem sie abgeleitet ist.

8. Ein Verfahren nach einem der vorhergehenden Ansprüche, worin die genannte 5'-Flankensequenz jede des ADH1-Strukturgens ist.

9. Ein Verfahren nach einem der vorhergehenden Ansprüche, worin die genannte Sequenz für die phenotypische Selektion in Hefe das TRP1-Gen ist.

10. Ein Verfahren nach einem der vorhergehenden Ansprüche, umfassend die Schaffung eines bakteriellen Replikationsursprungs und einen oder mehrere Sequenz(en) für die phenotypische Selektion in Bakterien und Anwendung derselben für die Verstärung von DNS in Bakterien in Zwischenstadien des Vektoraufbaus.

11. Ein Verfahren nach einem der vorhergehenden Ansprüche, zusätzlich umfassend das Legieren einer genannten exogenen Strukturgenkodierung für ein biokompetentes Polypeptid im Vektor an der genannten Einfügungsstelle, um dieses unter der Steuerung des genannten Promotors transkribierbar und von einem Startsignal translatierbar zu machen.

12. Ein Verfahren nach Anspruch 11, worin das genannte exogene Gen ein biokompetentes Polypeptid kodiert.

13. Ein Verfahren nach Anspruch 11, worin das genannte exogene Gen ein Säugetier-Polypeptid kodiert.

14. Ein Verfahren nach Anspruch 13, worin das genannte Säugetier-Polypeptid aus normalen und hybriden menschlichen Interferonen, menschlichem Proinsulin, die A- und B-Ketten von menschlichem Insulin, menschlichem Wachstumshormon, Somatostatin und Thymosiin Alpha 1 gewählt wird.

15. Ein Verfharen nach Anspruch 13, worin das genannte Säugetier-Polypeptid das von Leukocyt-Interferon D ist.

16. Ein Verfahren zur Bildung eines Hefetransformanten, der fähig ist, ein exogenes Gen zu exprimieren, um ein Polypeptid zu erzeugen, umfassend das Transformieren eines geeigneten Hefestammes mit einem rekombinanten DNS-Vektor nach einem der Ansprüche 11—15.

17. Ein Verfahren nach Anspruch 16, worin das genannte Polypeptid nicht für das Wachstum des Transformanten erforderlich ist.

18. Ein Verfahren nach Anspruch 16 oder 17, worin in dem genannten rekombinanten DNS-Vektor das phenotypische Gen für die Selektion in Hefe eine Mutation komplementiert, die vom Hefestamm getragen wird.

19. Ein Verfahren nach einem der Ansprüche 16, 17, und 18 worin die Hefe zur Gattung Saccharomyces gehört.

20. Ein Verfahren nach Anspruch 19, worin die Hefe zur Species Saccharomyces cerevisiae gehört.

21. Ein Verfahren nach Anspruch 20, worin die Hefe der Stamm Saccharomyces cerevisiae RH 218 ist.

22. Ein Verfahren zur Herstellung eines heterologen Polypeptids, das das Kultivieren eines Hefestammes nach einem der Ansprüche 16—21 und das Trennen des genannten biokompetenten Polypeptides aus dek Kulturmedium umfaßt.

23. Ein Verfahren zur Herstellung eines gewünschten heterologen Polypeptids in Hefe durch Kultivieren eines Hefestammes, der mit einem rekombinanten DNS-Expressionsvektor transformiert ist, der in dem genannten Hefestamm replizierbar ist, dadurch gekennzeichnet, daß der Vektor eine exogene DNS-Sequenzkodierung für das Polypeptid transkriptionell stromabwärts von einer 5'-Flankensequenz eines Hefesturgens enthält, das einen Promotor enthält, der funktionell in dem genannten Hefestamm ist, und daß ein Translationsauslösungssignal zwischen dem genannten Promotor und der exogenen Kodierungssequenz vorgesehen ist, sodaß die exogene Sequenz vom genennten Promotor transkribiert und vom genannten Translationsauslösungssignal translatiert wird.

24. Ein Verfahren nach Anspruch 23, worin der Expressionsvektor eine Transkriptionsterminations-sequenz stromabwärts von der genannten exogenen Kodierungssequenz aufweist.

25. Ein Verfahren nach Anspruch 23 oder 24, worin das Translationsauslösungssignal an der oder

stromaufwärts von der Stelle gelegen ist, die normalerweise von dem Translationsauflösungssignal des Hefestrukturgens eingenommen wird, von dem die genannte 5'-Flankensequenz abgeleitet ist.

Revendications pour les Etats Contractants: BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Vecteur d'ADN approprié à une utilisation dans l'expression de gènes exogènes dans une levure, comprenant une séquence qui est réplicable dans la levure, une séquence flanquant 5' d'un gène de structure de la levure comprenant un promoteur, un site en aval de ladite séquence flanquant 5' dans la direction de transcription pour l'insertion d'un gène de structure codant pour un polypeptide habituellement exogène à la levure afin de pouvoir être transcrit sous le contrôle dudit promoteur et translaté d'un signal de départ, et une séquence permettant la sélection phénotypique des transformants de la levure.

2. Vecteur d'ADN selon la revendication 1 qui comprend de plus une séquence de terminaison de transcription en aval dudit site d'insertion.

3. Vecteur d'ADN selon la revendication 2 où ladite séquence de terminaison de transcription est une séquence flanquant d'un gène de levure.

4. Vecteur d'ADN selon la revendication 3 où ledit gène de levure produit la sélection phénotypique de transformants de levure.

5. Vecteur d'ADN selon l'une quelconque des revendications précédentes où ladite séquence flanquant 5' manque d'un signal de départ de translation en amont dudit site d'insertion par rapport à la direction de translation.

6. Vecteur d'ADN selon l'une quelconque des revendications précédentes où ladite séquence flanquant 5' manque du signal de départ de translation du gène d'où elle est dérivée.

7. Vecteur d'ADN selon la revendication 6 où ledit site d'insertion se trouve à ou en amont, par rapport à la direction de translation, de la position normalement occupée par le signal de départ de translation du gène d'où il est dérivé.

8. Vecteur d'ADN selon l'une quelconque des revendications précédentes où ladite séquence flanquant 5' est celle du gène de structure ADH1.

9. Vecteur d'ADN selon l'une quelconque des revendications précédentes où ladite séquence pour la sélection phénotypique dans la levure est le gène TRP1.

10. Vecteur d'ADN selon l'une quelconque des revendications précédentes qui comprend, de plus, une origine bactérienne de réplication et une ou plusieurs séquences pour la sélection phénotypique dans les bactéries.

11. Vecteur d'ADN recombinant à utiliser pour exprimer un gène exogène de structure dans une souche appropriée de levure, comprenant un vecteur d'ADN selon l'une quelconque des revendications précédentes et ledit gène exogène inséré audit site afin de pouvoir être transcrit sous le contrôle dudit promoteur et translaté à partir d'un signal de départ.

12. Vecteur d'ADN recombinant selon la revendication 11 où ledit gène exogène code un polypeptide biocompétent.

13. Vecteur d'ADN recombinant selon la revendication 11 où ledit gène exogène code un polypeptide mammifère.

14. Vecteur d'ADN recombinant selon la revendication 13 où ledit polypeptide mammifère est choisi parmi des interférons humains normaux et hybrides, la proinsuline humaine, les chaînes A et B de l'insuline humaine, l'hormone de la croissance humaine, la somatostatine et la thymosine alpha 1.

15. Vecteur d'ADN recombinant selon la revendication 13 où ledit polypeptide mammifère est celui de l'interféron D de leucocyte.

16. Souche de levure transformée par un vecteur d'ADN recombinant selon l'une quelconque des revendications 11 à 15 capable d'exprimer ledit gène exogène pour produire un polypeptide.

17. Souche de levure transformée selon la revendication 16 où ledit polypeptide n'est pas requis pour la croissance de transformant.

18. Souche de levure transformée selon la revendication 16 ou la revendication 17 où, dans ledit vecteur d'ADN recombinant, le gène phénotypique pour la sélection dans la levure complète une mutation supportée par la souche de la levure.

19. Souche de levure transformée selon l'une quelconque des revendications 16, 17 et 18 où la levure est du genre Saccharomyces.

20. Souche de levure transformée selon la revendication 19 où la levure est de l'espèce Saccharomyces cerevisiae.

21. Souche de levure transformée selon la revendication 20 où la levure est la souche Saccharomyces cerevisiae RH 218.

22. Méthode de formation d'une souche de levure transformée selon l'une quelconque des revendications 16 à 21, laquelle méthode consiste à:

(a) prévoir un vecteur de transfert d'ADN réplicable dans les bactéries et la levure et des gènes pour la sélection phénotypique des transformants bactériens et de la levure;

(b) prévoir un fragment d'ADN comprenant un gène de structure codant un polypeptide ordinairement exogène à la levure;

(c) prévoir un fragment d'ADN comprenant un promoteur de la levure génétiquement distinct dudit gène de structure exogène.

(d) insérer les fragments de (b) et (c) dans ledit vecteur de transfert avec signaux positionnés de manière appropriée de départ de translation et d'arrêt pour ledit gène exogène pour former un vecteur d'ADN recombinant dans lequel la transcription dudit gène exogène est sous la contrôle dudit promoteur et la translation est impartie dudit signal de départ, la réplication bactérienne et la sélection phénotypique étant employées pour l'amplification de l'ADN dans la bactérie en des stades intermédiaires dans la construction du vecteur; et

(e) la transformation d'une souche de levure appropriée par le vecteur résultant de manière que la gène exogène puisse être exprimé dans la transformant.

23. Méthode de production d'un polypeptide hétérologue qui comprend la mise en culture d'une souche de levure selon l'une quelconque des revendications 16 à 22 et l'isolement dudit polypeptide biocompétent du milieu de culture.

24. Méthode de production d'un polypeptide hétérologue souhaité dans une levure par mise en culture d'une souche de levure transformée par un vecteur d'expression d'ADN recombinant réplicable dans ladite souche de levure, caractérisée en ce que le vecteur contient une séquence d'ADN exogène codant pour la polypeptide par transcription en aval d'une séquence flanquant 5' d'un gène de structure de levure contenant un promoteur qui est fonctionnel dans ladite souche de levure, et un signal d'initiation de translation entre ledit promoteur et la séquence codante exogène, de manière qui la séquence exogène soit transcrite dudit promoteur et translatée dudit signal d'initiation de translation.

25. Méthode selon la revendication 24 où le vecteur d'expression comprend de plus une séquence de terminaison de transcription en aval de ladite séquence codante exogène.

26. Méthode selon la revendication 24 ou la revendication 25 où le signal d'initiation de translation se trouve à ou en amont de la position normalement occupée par le signal d'initiation de translation du gène de structure de la levure d'où est dérivée ladite séquence flanquant 5'.

Revendications pour l'Etat Contractant: AT

1. Méthode de formation d'un vecteur d'ADN approprié à une utilisation pour l'expression de gènes exogènes dans une levure, laquelle méthode comprend la ligature des fragments d'ADN ensemble pour former un vecteur comprenant une séquence qui est réplicable dans la levure, une séquence flanquant 5' d'un gène de structure de levure comprenant un promoteur, un site en aval de ladite séquence flanquant 5' dans la direction de transcription pour insertion d'un gène de structure codant pour un polypeptide habituellement exogène à la levure de manière à pouvoir être transcrit sous le contrôle dudit promoteur et translaté à partir d'un signal de départ, et une séquence permettant la sélection phénotypique des transformants de la levure.

2. Méthode selon la revendication 1 qui consiste à prévoir une séquence de terminaison de transcription en aval dudit site d'insertion.

3. Méthode selon la revendication 2 où ladite séquence de terminaison de transcription est une séquence flanquante d'un gène de levure.

4. Méthode selon la revendication 3 où ledit gène de levure produit la sélection phénotypique des transformants de la levure.

5. Méthode selon l'une quelconque des revendications précédentes où ladite séquence flanquant 5' manque d'un signal de départ de translation en amont dudit site d'insertion par rapport à la direction de translation.

6. Méthode selon l'une quelconque des revendications précédentes où ladite séquence flanquant 5' manque du signal de départ de translation du gène d'où elle est dérivée.

7. Méthode selon la revendication 6 où ledit site d'insertion se trouve à ou en amont, par rapport à la direction de translation, de la position normalement occupée par le signal de départ de translation du gène d'où il est dérivé.

8. Méthode selon l'une quelconque des revendications précédentes où ladite séquence flanquant 5' est celle du gène de structure ADH1.

9. Méthode selon l'une quelconque des revendications précédentes où ladite séquence pour la sélection phénotypique dans une levure est le gène TRP1.

10. Méthode selon l'une quelconque des revendications précédentes qui consiste à prévoir une origine bactérienne de réplication et une ou plusieurs séquences pour la sélection phénotypique dans les bactéries, et à l'utiliser pour l'amplification de l'ADN dans des bactéries en des stades intermédiaires dans la construction du vecteur.

11. Méthode selon l'une quelconque des revendications précédentes qui comprend la ligature supplémentaire, dans le vecteur, audit site d'insertion, dudit gène exogène de structure codant pour un polypeptide biocompétent afin de pouvoir être transcrit sous le contrôle dudit promoteur et translaté à partir d'un signal de départ.

12. Méthode selon la revendication 11 où ledit gène exogène code un polypeptide biocompétent.

13. Méthode selon la revendication 11 où ledit gène exogène code un polypeptide mammifère.

14. Méthode selon la revendication 13 où ledit polypeptide mammifère est choisi parmi les interférons

humains normaux et hybrides, la proinsuline humaine, les chaînes A et B et l'insuline humaine, l'hormone de la croissance humaine, la somatostatine et la thymosine alpha 1.

15. Méthode selon la revendication 13 où ledit polypeptide mammifère est celui de l'interféron D de leucocyte.

16. Méthode de formation d'un transformant de levure capable d'exprimer un gène exogène pour produire un polypeptide qui consiste à transformer une souche appropriée de levure par un vecteur d'ADN recombinant selon l'une quelconque des revendications 11 à 15.

17. Méthode selon la revendication 16 où ledit polypeptide n'est pas requis pour la croissance du transformant.

18. Méthode selon la revendication 16 ou la revendication 17 où, dans ledit vecteur d'ADN recombinant, le gène phénotypique pour la sélection dans la levure complète une mutation supportée par la souche de levure.

19. Méthode selon l'une quelconque des revendications 16, 17 ou 18 où la levure est du genre Saccharomyces.

20. Méthode selon la revendication 19 où la levure est de d'espèce Saccharomyces cerevisiae.

21. Méthode selon la revendication 20 où la levure est la souche Saccharomyces cerevisiae RH 218.

22. Méthode de production d'un polypeptide hétérologue qui comprend la mise en culture d'une souche de levure selon l'une quelconque des revendications 16 à 21 et l'isolement dudit polypeptide biocompétent du milieu de culture.

23. Méthode de production d'un polypeptide hétérologue souhaité dans une levure par mise en culture d'une souche de levure transformée par un vecteur d'expression d'ADN recombinant réplicable dans ladite souche de levure, caractérisée en ce que le vecteur contient une séquence d'ADN exogène codant pour le polypeptide en aval par transcription d'une séquence flanquant 5' d'un gène de structure de levure contenant un promoteur qui est fonctionnel dans ladite souche de levure, et un signal d'initiation de translation entre ledit promoteur et la séquence exogène codante, de manière que la séquence exogène soit transcrite dudit promoteur et translatée dudit signal d'initiation de translation.

24. Méthode selon la revendication 23 où le vecteur d'expression comprend une séquence de terminaison de transcription en aval de ladite séquence codante exogène.

25. Méthode selon la revendication 23 ou 24 où le signal d'initiation de translation se trouve à ou en amont de la position normalement occupée par le signal d'initiation de translation du gène de structure de la levure d'où est dérivée ladite séquence flanquant 5'.

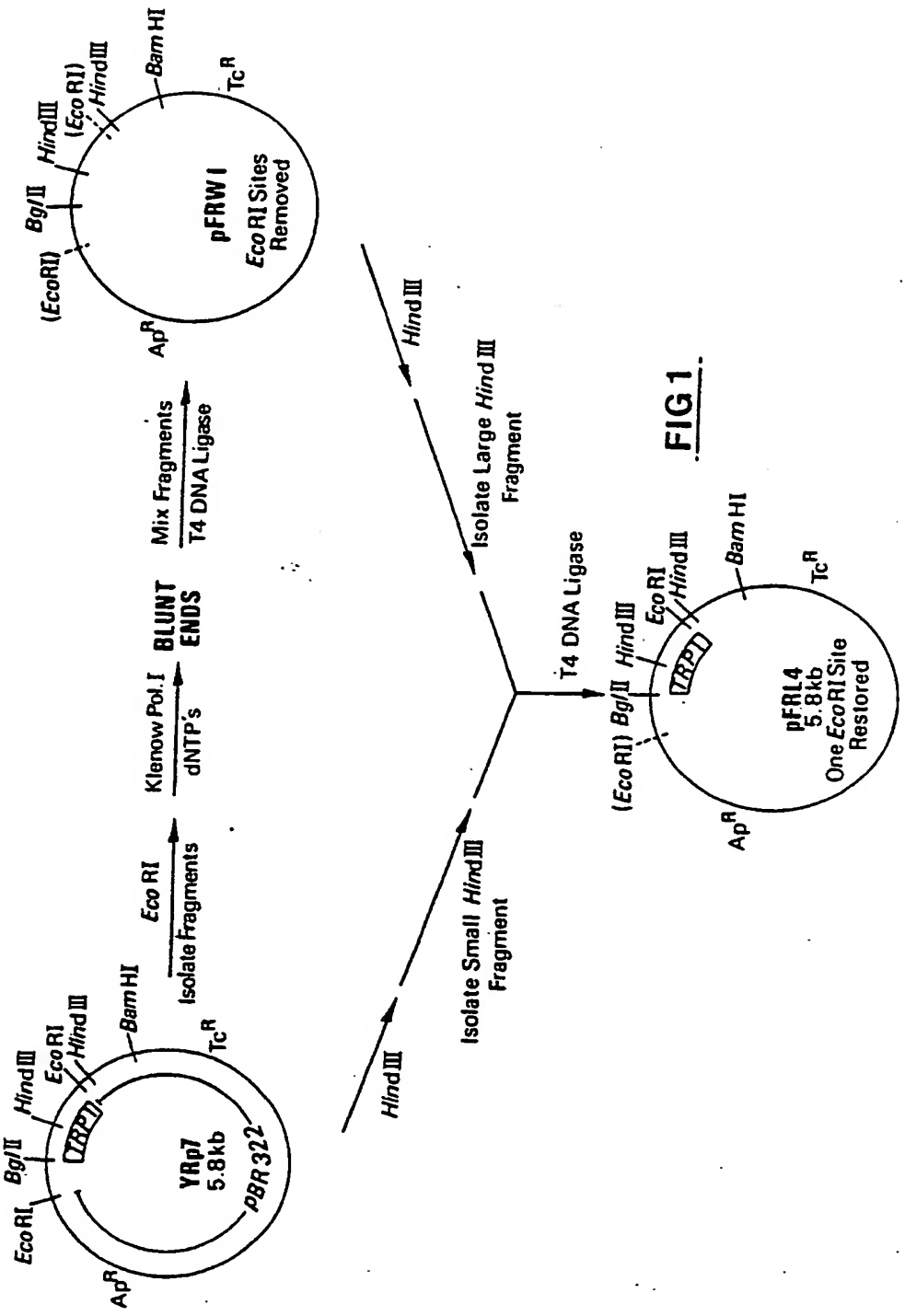
EcoRI
Bgl II
Hind III
EcoRI
Hind III
Bam HI
YRp7
5.8kb
pBR322
TRP1
Ap^R
Tc^R
Isolate Fragments
Eco RI
Klenow Pol.I
dNTP's
BLUNT ENDS
Mix Fragments
T4 DNA Ligase
(EcoRI)
Bgl II
Hind III
(EcoRI)
Hind III
Bam HI
pFRW I
Eco RI Sites Removed
Ap^R
Tc^R
Hind III
Isolate Small Hind III Fragment
Hind III
Isolate Large Hind III Fragment
T4 DNA Ligase
(EcoRI)
Bgl II
Hind III
Eco RI
Hind III
Bam HI
TRP1
pFRL4
5.8kb
One Eco RI Site Restored
Ap^R
Tc^R

FIG 1

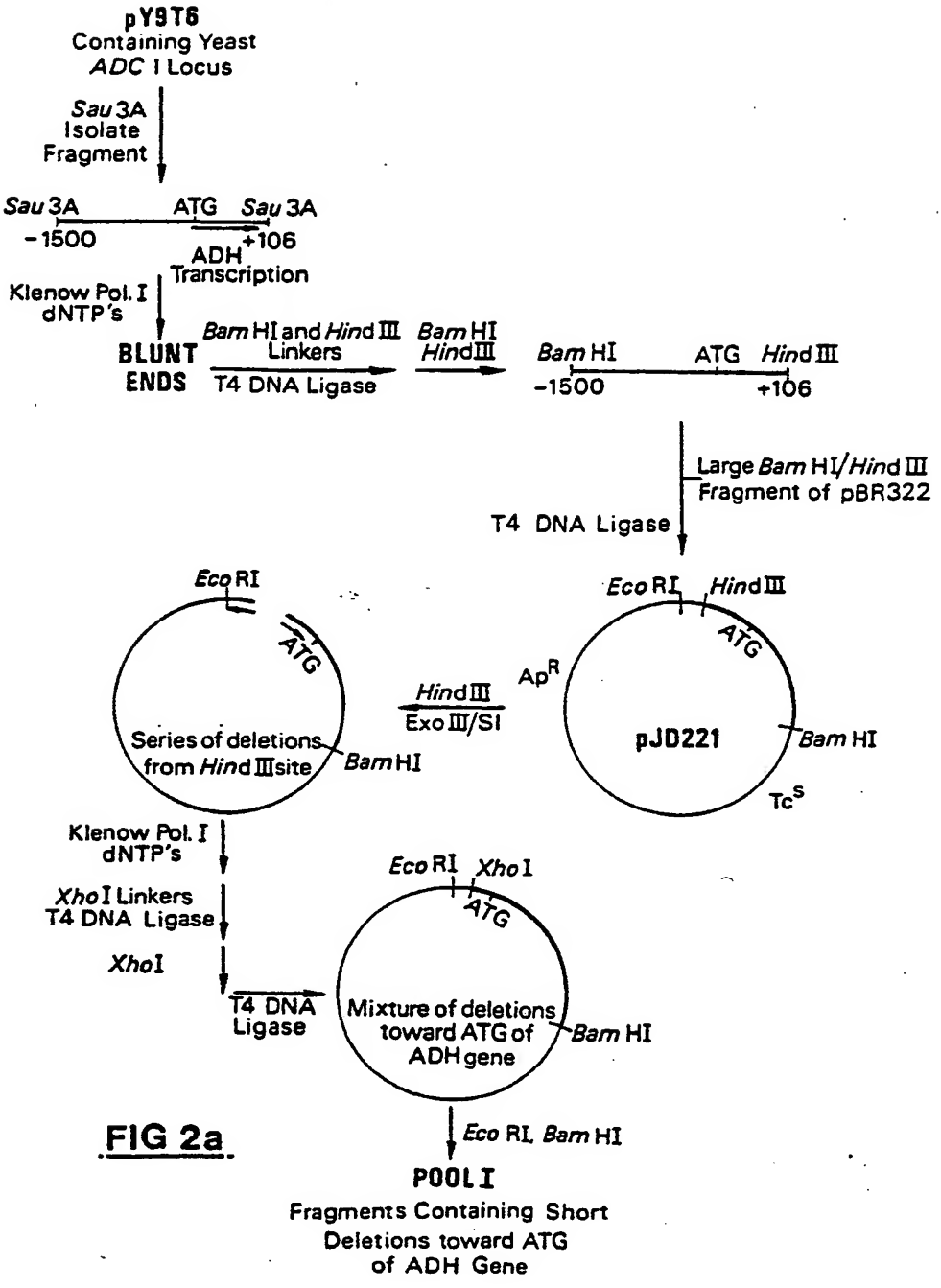
pY9T6
Containing Yeast
ADC I Locus
Sau 3A
Isolate
Fragment
Sau 3A
ATG
Sau 3A
-1500
+106
ADH
Transcription
Klenow Pol. I
dNTP's
BLUNT
ENDS
Bam HI and Hind III
Linkers
T4 DNA Ligase
Bam HI
Hind III
Bam HI
ATG
Hind III
-1500
+106
Large Bam HI/Hind III
Fragment of pBR322
T4 DNA Ligase
Eco RI
ATG
Series of deletions
from Hind III site
Bam HI
Hind III
Exo III/SI
Eco RI
Hind III
ATG
Ap^R
pJD221
Bam HI
Tc^S
Klenow Pol. I
dNTP's
Xho I Linkers
T4 DNA Ligase
Xho I
T4 DNA
Ligase
Eco RI
Xho I
ATG
Mixture of deletions
toward ATG of
ADH gene
Bam HI
Eco RI, Bam HI
POOL I
Fragments Containing Short
Deletions toward ATG
of ADH Gene

FIG 2a

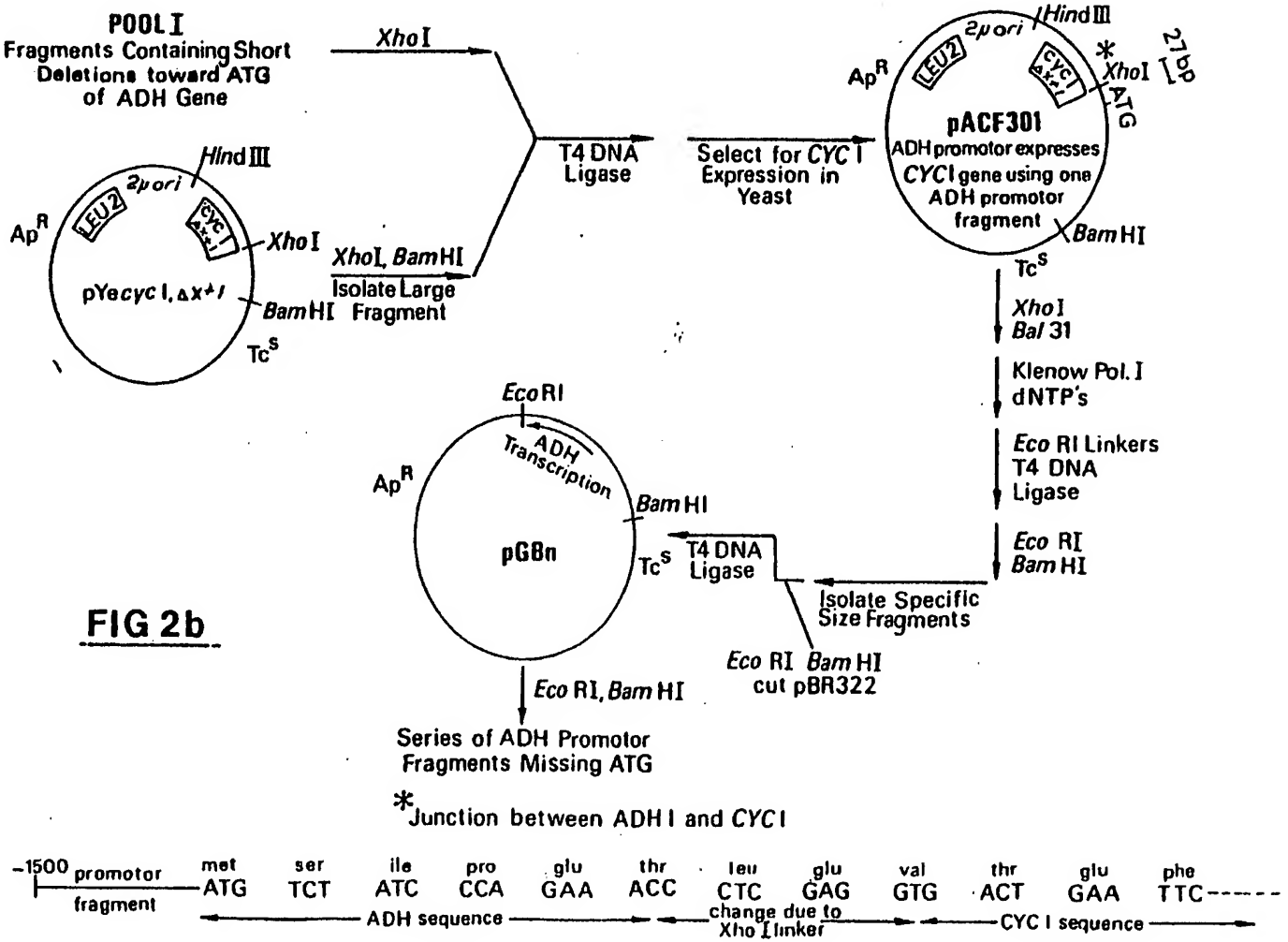

FIG 2b

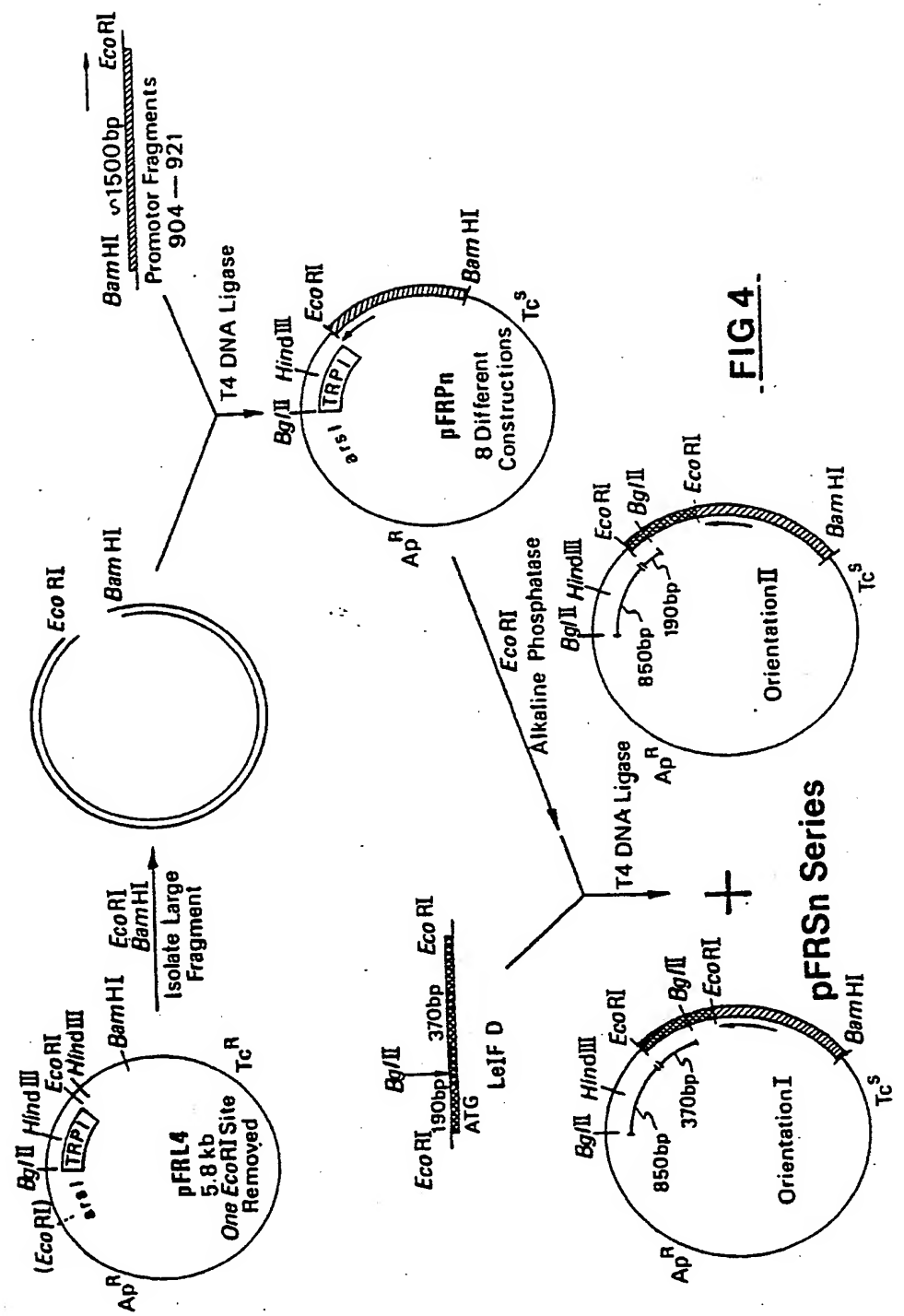
pFRL4
5.8 kb
One EcoRI Site
Removed
Isolate Large
Fragment
T4 DNA Ligase
Promotor Fragments
904 — 921
pFRPn
8 Different
Constructions
Alkaline Phosphatase
LeIF D
ATG
Orientation I
Orientation II
pFRSn Series
T4 DNA Ligase
370bp
190bp
850bp

FIG 4

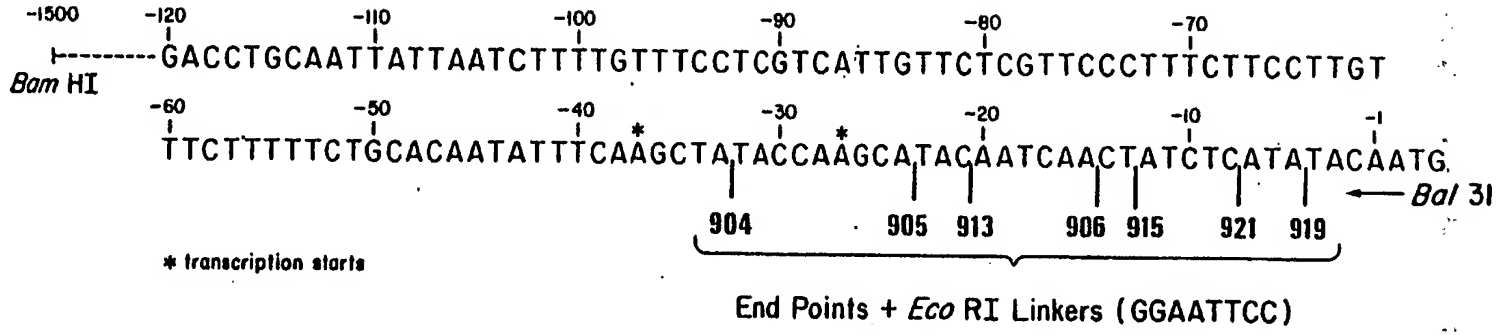

FIG 3